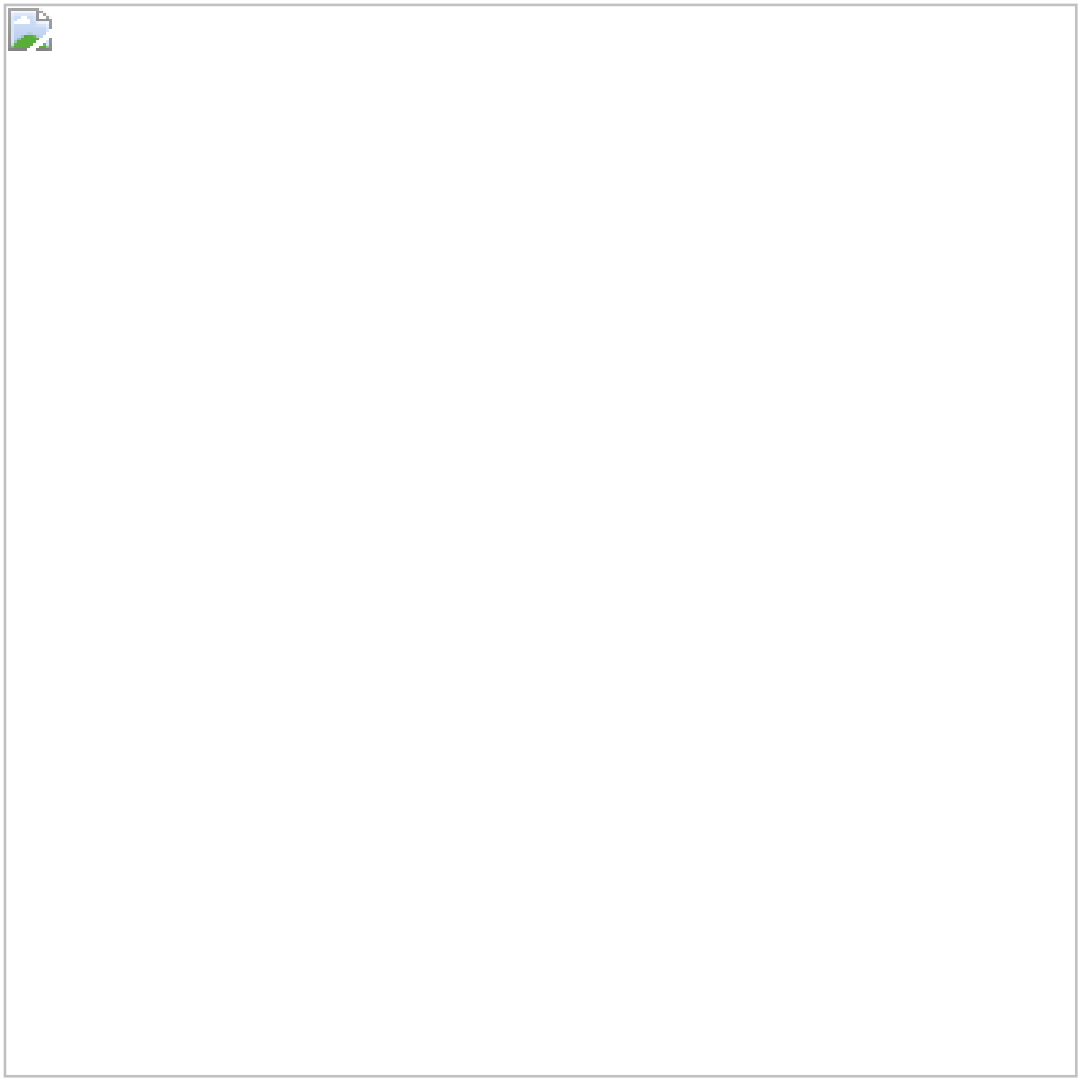

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Branched-Chain Amino Acid Metabolism in *Arabidopsis thaliana*

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Valine, leucine and isoleucine form the small group of branched-chain amino acids (BCAAs) classified by their small branched hydrocarbon residues. Unlike animals, plants are able to de novo synthesize these amino acids from pyruvate, 2-oxobutanoate and acetyl-CoA. In plants, biosynthesis follows the typical reaction pathways established for the formation of these amino acids in microorganisms. Val and Ile are synthesized in two parallel pathways using a single set of enzymes. The pathway to Leu branches of from the final intermediate of Val biosynthesis. The formation of this amino acid requires a three-step pathway generating a 2-oxoacid elongated by a methylene group. In *Arabidopsis thaliana* and other Brassicaceae, a homologous three-step pathway is also involved in Met chain elongation required for the biosynthesis of aliphatic glucosinolates, an important class of specialized metabolites in Brassicaceae. This is a prime example for the evolutionary relationship of pathways from primary and specialized metabolism. Similar to animals, plants also have the ability to degrade BCAAs. The importance of BCAA turnover has long been unclear, but now it seems apparent that the breakdown process might by relevant under certain environmental conditions. In this review, I summarize the current knowledge about BCAA metabolism, its regulation and its particular features in *Arabidopsis thaliana.*

INTRODUCTION

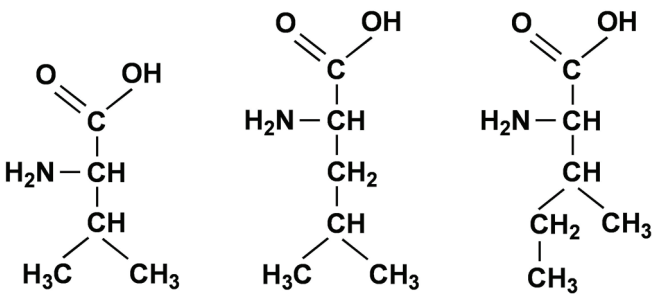
Valine, leucine and isoleucine form the small group of branched-chain amino acids (BCAAs). They are classified by their small branched hydrocarbon residues responsible for the aliphatic character of these molecules (Figure 1). As a result of their aliphatic nature, BCAAs are predominantly found in membrane-spanning protein domains.

The metabolism of these amino acids shows a number of similarities between organisms of the different kingdoms of life. However, there are also striking differences concerning the capabilities to synthesize or degrade branched-chain amino acids. Beyond these differences BCAAs have other unique functions and features in the different groups of organisms.

Animals, including humans, are not able to de novo synthesize BCAAs. Therefore they have to take up these amino acids with their diets or depend on other sources such as symbiotic bacteria (Akman Gunduz and Douglas, 2009). Homeostasis of BCAAs seems to be well balanced in animals, probably since these amino acids, predominantly Leu have additional important functions. Thus superfluous BCAAs are degraded and the importance of the degradation becomes evident by the severe diseases linked to disorders in BCAA breakdown. Just to mention one example, impaired branched-chain keto acid dehydrogenase activity leads to the Maple Syrup Urine Disease, which can manifest in neurological degeneration (Chuang et al., 2006).

In animals and humans, Leu is an important signaling molecule, which regulates food intake, stimulates translation and is involved in triggering autophagy. These functions involve the mTOR and S6K1 signaling pathway, which has a crucial function in the nutrient sensing pathway (Cota et al., 2006; Kimball and Jefferson, 2006a, b; Woods et al., 2008). Although a few components of these signaling pathways are conserved in *Arabidopsis thaliana*, analogous functions of Leu or the other BCAAs have not yet been described in plants.

Bacteria, archaea, fungi and plants are able to synthesize BCAAs. Apart from the threonine independent citramalate pathway in some prokaryotes, in which Ile is formed from pyruvate and acetyl-CoA (Wu et al., 2009), the biosynthesis of BCAAs follows a common scheme, as described in this article for plants. Research



L-valine *L*-leucine *L*-isoleucine

Figure 1. Structures of the three branched-chain amino acids.

interest in the biosynthesis of BCAAs in plants has mainly been driven by the role of some biosynthetic enzymes as targets for herbicides. Acetohydroxyacid synthase in particular is inhibited by several classes of commercially important herbicides (Wittenbach and Abell, 1999; Tan et al., 2006). While all enzymatic activities required for BCAA biosynthesis have been detected in plants, not all enzymes and genes involved in the formation of these amino acids have been characterized in detail. However, progress in this field has benefited enormously from complete genome sequences. The thorough bioinformatic analysis of these sequences identified many candidate genes and facilitated the targeted analysis of several metabolic enzymes. In these studies, the model species *Arabidopsis thaliana* with its well developed scientific infrastructure played a unique role. In this context, investigation of *Arabidopsis thaliana* as a Brassicaceae species has been particularly relevant for analyses of the Leu biosynthesis and the methionine chain elongation pathway, two evolutionary closely related pathways of primary and specialized metabolism (formerly known as secondary metabolism).

Like animals, plants posses the capability to degrade BCAAs. In recent years, many enzymes required for the breakdown of these amino acids have been identified and *Arabidopsis thaliana* played an important role in the characterization of these proteins. However, our knowledge of BCAA degradation and its importance for plant metabolism is far from complete and this model species will be the ideal organism for future studies.

BIOSYNTHESIS OF BRANCHED-CHAIN AMINO ACIDS

A unique feature of BCAA biosynthesis is that Val and Ile are synthesized in two parallel pathways. This is achieved with a single set of four enzymes, which catalyze the reactions towards the formation of these amino acids with different substrates (Figure 2). These enzymes are: acetohydroxyacid synthase (AHAS, EC 4.1.3.18), ketolacid reductoisomerase (KARI, EC 1.1.1.86), dihydroxyacid dehydratase (DHAD, EC 4.2.1.9) and branched-chain aminotransferase (BCAT, EC 2.6.1.42) (Singh and Shaner, 1995; Singh, 1999). An exception is threonine deaminase (TD, EC 4.2.1.16), which catalyzes the deamination and dehydratation of Thr. This reaction yields ammonia and 2-oxobutanoate (α-ketobutyrate), the latter being one of the initial substrates for the synthesis of Ile. The pathway towards Leu starts out from 2-oxoisovalerate, the last intermediate that is transaminated to form Val.

Several older studies, including ones involving subcellular localization by GFP fusion proteins, and recent proteomic approaches revealed that branched-chain amino acids are synthesized in chloroplasts (Ellerstrom et al., 1992; Diebold et al., 2002; Zybailov et al., 2008).

Threonine Deaminase

Threonine deaminase (TD, EC 4.2.1.16, also known as threonine dehydratase, Figure 2) catalyzes the first reaction in the canonical pathway for the biosynthesis of Ile. This reaction produces ammonia and 2-oxobutanoate, which together with pyruvate are the substrates for AHAS (see below). In *Arabidopsis thaliana*, TD is encoded by a single locus (*OMR1*, At3g10050). This gene has been identified in a screening for L-O-methylthreonine-resistant mutants, which accumulate up to 20-fold more Ile than wild-type plants (Mourad and King, 1995). In the homozygous mutants (*omr1-1/omr1-1*), TD is insensitive to feedback inhibition by Ile due to two amino acid substitutions at the carboxy-terminal end (R499C and R544H). TD normally controls the flux of Thr into Ile biosynthesis (Singh and Shaner, 1995; Singh, 1999). The formation of 2-oxobutanoate from Thr also links Ile biosynthesis with the metabolism of the Met. Thus Ile together with Thr, Met and Lys form the group of aspartate-derived amino acids (Jander and Joshi, 2009, 2010).

Beside TD, plants seem to have an alternative way to recruit 2-oxobutanoate. A number of reports provided indirect evidence for a metabolic flux from Met to Ile (Zeh et al., 2001). For instance in potato tubers with antisense-inhibited threonine synthase (TS, EC 4.2.3.1, At4g29840), Met levels increased by a factor of 30 accompanied by an elevated accumulation of Ile. Since no apparent change was seen in Thr content, the increase of Ile occurred in an Thr-independent way (Zeh et al., 2001). Likewise overexpression of cystathionine γ-synthase (CgS, EC 2.5.1.48, At3g01120) increased Met and Ile levels in potato tubers (Dancs et al., 2008). It was suggested that the increase of Ile is directly associated with the activity of methionine γ-lyase (MGL, 4.4.1.11, At1g64660), which produces 2-oxobutanoate, methanethiol and ammonia from Met. In *Arabidopsis thaliana* cell suspension culture Met-derived 2-oxobutanoate can be incorporated into Ile (Rebeille et al., 2006) and very recently the importance of MGL for Ile biosynthesis was investigated in plants (Joshi and Jander, 2009). This study demonstrated that TD knockdown plants (*omr1-9*) accumulate significantly lower levels of branched-chain amino acids than wild type. Only overexpression of MGL rescued this phenotype, demonstrating that the natural level of MGL activity is not sufficient to compensate the lower TD activities in *omr1-9*. This observation strongly suggests that MGL and TD have overlapping functions in Ile biosynthesis, however, MGL is of secondary importance under normal growth conditions. In addition, TD competes with two Thr aldolases (EC 4.1.2.5. THA1, At1g08630 and THA2, At3g04520) for a common substrate pool. Rescue of *tha1/tha2* double mutants by overproduction of feedback-insensitive TD demonstrates that Thr aldolases are not essential for Gly formation in *Arabidopsis thaliana* (Joshi et al., 2006). Interestingly, TD and other jasmonate-inducible plant enzymes have been found to accumulate in the midgut of herbivorous insect larvae. There, TD is responsible for the degradation of Thr, which lowers the availability of this essential amino acid for the herbivore (Chen et al., 2005). In the insect the breakdown seems to be enhanced by the proteolytic removal of the C-terminus, which prevents feedback inhibition of Ile. In addition, TD activity is important for Ile conjugated JA signaling (JA-Ile) at least in Solanaceae. This signaling pathway is crucial for promoting plants resistance to herbivores. Mild silencing of TD in *Nicotiana attenuata* leads to an ample susceptibility to *Manduca sexta*, and resistance can be restored by addition of Ile or JA-Ile (Kang et al., 2006).

Acetohydroxyacid Synthase

Acetohydroxyacid synthase (AHAS, EC 4.1.3.18, also known as acetolactate synthase ALS, Figure 2) catalyzes the first step in

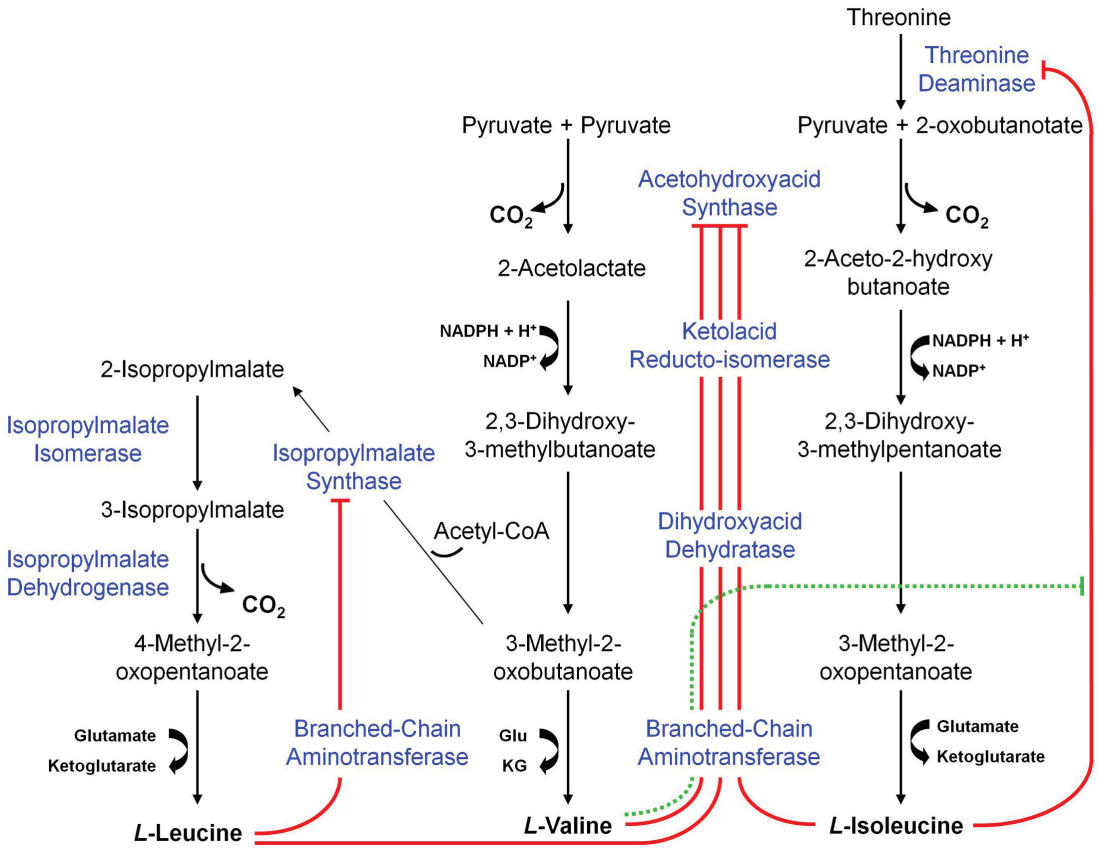


Figure 2. Key enzymes (blue) and metabolites (black) of branched-chain amino acid biosynthesis. Allosteric inhibition is indicated by red lines. The restoring effect of Val on threonine deaminase inhibition by Ile is depicted as a dotted green line.

the parallel pathways towards Val and Leu and towards Ile. It catalyzes the conversion of two molecules of pyruvate into 2-acetolactate (Val, Leu) or one molecule of pyruvate and one molecule of 2-oxobutanoate into 2-aceto-2-hydroxybutyrate (Ile) (Singh, 1999; Duggleby et al., 2008). The enzyme requires thiamine diphosphate (ThDP) as essential cofactor. ThDP is anchored to the active site of the enzyme by a divalent metal ion such as Mg^{2+}. In addition, AHAS needs flavin adenine dinucleotide (FAD) as a third cofactor. The presence of this cofactor in AHAS seems to be an evolutionary relic (Duggleby et al., 2008).

Like in bacteria and yeast, AHAS from plants is composed of separate catalytic and regulatory subunits, whose reconstitution *in vitro* results in a heterodimer with an activity at least 5-fold higher than the catalytic subunit alone (Hershey et al., 1999; Lee and Duggleby, 2001). In *Arabidopsis thaliana* the catalytic subunit is encoded by At3g48560, while At2g31810 and probably also At5g16290 encode regulatory subunits. The regulatory protein is necessary for AHAS to be inhibited by branched-chain amino acids. It exhibits a structure similar to TD, suggesting that these proteins are evolutionarily related (Lee and Duggleby, 2001).

AHAS is probably the best-studied enzyme of BCAA metabolism since it is the target for commercially successful herbicides. There are five classes of AHAS-inhibiting herbicides: imidazolinones, sulfonylureas, triazolopyrimidines, pyrimidinylthio (to oxy)-benzoates and sulfonylamino-carbonyltriazolinones. Most of the commercialized AHAS inhibitors belong to the sulfonylurea class (Wittenbach and Abell, 1999; Tan et al., 2006). As mentioned above, the interaction of *Arabidopsis thaliana* AHAS with several members of these herbicide families has been analyzed by co-crystallization. The different herbicide molecules do not mimic the substrates of AHAS, but bind to the substrate access channel thereby blocking substrate entry to the active site (McCourt et al., 2006; Duggleby et al., 2008). This mode of action, which is independent from the active site, explains the relatively rapid development of weeds resistant to AHAS-inhibiting herbicides. At least 17 amino acid residues in AHAS have been identified in bacteria, fungi and plant where mutations result into herbicide resistance. In *Arabidopsis thaliana*, several herbicide-resistant mutants with mutated AHAS were isolated and characterized (Hattori et al., 1995; Mourad et al., 1995). Among them mutations of W574 give rise to strong resistance to four classes of AHAS-inhibiting herbicides. As shown by the crystallization studies this Trp residue interacts with the heterocyclic ring of sulfonylureas and consequently mutation of the residue prevents this interac-

tion. In addition, mutation of W574 could change the contour of the herbicide binding site resulting in a less complementary fit for the sulfonylureas.

Arabidopsis thaliana AHAS has been co-crystallized with various sulfonylurea and imidazolinone herbicides (McCourt et al., 2006). In the presence of these herbicides AHAS crystallizes as a tetramer. This finding did not fit with previous gel filtration studies, in which this enzyme eluted at an apparent molecular mass of 110 kDa indicating that the catalytic subunit exists as a dimer. However, complexes formed between the regulatory and the catalytic subunits have an apparent mass of about 500 kDa suggesting that there are four regulatory and four catalytic proteins in this assembly (Duggleby et al., 2008). Further insights into structure of *Arabidopsis thaliana* AHAS might be obtained from crystals of the catalytic subunit without herbicide or from co-crystallization of the catalytic and the regulatory subunits.

Ketolacid Reductoisomerase

The second step in the parallel pathways towards Val and Leu or Ile is catalyzed by the ketolacid reductoisomerase (KARI, EC 1.1.1.86), also known as acetohydroxyacid isomeroreductase (AHIR) (Singh, 1999; Dumas et al., 2001) (Figure 2). This enzyme catalyzes an unusual two-step reaction including an alkyl migration to form the intermediates 3-hydroxy-3-methyl-2-oxobutyrate from 2-acetolactate or 3-hydroxy-3-methyl-2-oxopentanoate from 2-aceto-2-hydroxybutyrate. This isomerization depends on Mg^{2+} and is followed by the reduction to the final dihydroxyacid products 2,3-dihydroxy-3-isovalerate or 2,3-dihydroxy-3-methylvalerate. The reduction depends on both NADPH and Mg^{2+}. KARI from plants shows an extremely high affinity towards Mg^{2+}, one of the strongest affinities ever reported between an enzyme and a metal ion (Singh, 1999; Dumas et al., 2001).

In *Arabidopsis thaliana*, KARI is encoded by At3g58610. This protein of about 59 kDa belongs to the class II type of this enzyme found in plants and some gram negative bacteria (Dumas et al., 1993). In the shorter class I form, found in most bacteria and fungi, 140 amino acids are missing in the N-terminal part of the C-terminal domain (Tyagi et al., 2005). Interestingly the additional 140 amino acids present in plants and gram negative bacteria, show no sequence similarity to each other. Crystallization of the class I-type KARI from *Pseudomonas aeruginosa* and of class II-type enzyme from *E. coli* revealed that the additional sequence in the long protein both in plants and *E. coli* is derived from a duplication of the C-terminal part present in the short class I form (Ahn et al., 2003; Tyagi et al., 2005).

KARI also attracted attention as a potential target for herbicides. The KARI inhibitors HOE 704 (2-dimethylphosphinoyl-2-hydroxy acetic acid) and IpOHA (N-hydroxy-N-isoproyloxamate) are nanomolar inhibitors, but display only minor herbicidal activity. The lack of potency in the field has been attributed to two reasons: (i) the inhibitors exhibit slow binding inhibition and (ii) they act as competitive inhibitors and the accumulation of the substrates prevents optimal effects (Leung and Guddat, 2009).

KARI from spinach has been crystallized in the presence of NADPH with either Mg^{2+} and IpOHA (Biou et al., 1997) or Mn^{2+} and 2-aceto-2-hydroxybutyrate (Thomazeau et al., 2000). More recently, crystal structures of the enzyme from rice in the presence of Mg^{2+} as well as in presence of both Mg^{2+} and NADPH have been determined (Leung and Guddat, 2009). These structural analyses consistently show that KARI from plants forms a dimer. The N-terminal domains of the identical subunits interact with NADPH. The C-terminal domain is almost entirely composed of α-helices and contributes to the formation of the metal ion and the substrate recognition sites but is also responsible for interaction between the monomers. The active site is situated at the interface between these domains and contains two divalent cations, five water molecules, one inhibitor or reaction product and the nicotinamide moiety of the NADPH (Dumas et al., 2001). The crystals obtained with different ligands also revealed that the N-domain seems to move with the state of catalysis. This suggests an overall mechanism of induced fit for catalysis and it has been speculated that in the free enzyme the N-domain is in constant motion. This would allow an easy access of the metal ions or NADPH but most likely also prevents crystallization of a plant KARI as a free enzyme (Leung and Guddat, 2009).

Furthermore KARI shows some additional unusual features. It has been identified as one of the few proteins containing a knot, the biological function of which is not clear (Taylor, 2000). In yeast, where BCAA biosynthesis occurs in the mitochondria, KARI (Ilv5p) has been found to bind and stabilize the organellar DNA. Why and how this is achieved by KARI is still unknown (Bateman et al., 2002).

Dihydroxyacid Dehydratase

In plants, little is known about the dihydroxyacid dehydratase (DHAD, EC 4.2.1.9). This enzyme catalyzes the dehydration of 2,3-dihydroxy-3-isovalerate or 2,3-dihydroxy-3-methylvalerate to the 2-oxo acids 3-methyl-2-oxobutanoate (3MOB, also know as ketoisovalerate, KIV) or 3-methyl-2-oxopentanoate (3MOP also know as ketomethylvalerate, KMV, Figure 2). These 2-oxo acids can now be converted into the corresponding amino acids or, as in the case of 3MOB, serve as substrate for the biosynthesis of Leu.

There are only few reports about this enzyme in plants, which date back to the eighties. DHAD has been purified from spinach and was found to contain an iron-sulfur cluster [2Fe-2S]. It was further shown that this enzyme is essential for BCAA biosynthesis (Singh, 1999). In *Arabidopsis thaliana*, DHAD is encoded by a single gene (At3g23940) identified by its similarity to bacterial DHAD sequences. Up to now, DHAD has not been investigated in this model species.

Isopropylmalate Synthase

3MOB, the last intermediate of Val biosynthesis, is also the initial substrate in the branch towards the biosynthesis of Leu (Figure 2). In this committed step, isopropylmalate synthase (IPMS, sometimes also abbreviated IMS, EC 2.2.3.13) catalyzes the condensation of 3MOB and acetyl-CoA, which yields 2-isopropylmalate.

Four genes encoding IPMS-like proteins are present in *Arabidopsis thaliana* ecotype Columbia-0 (Kroymann et al., 2001; Junk and Mourad, 2002; Field et al., 2004; Textor et al., 2004; de Kraker et al., 2007; Textor et al., 2007). Two of these genes, At5g23010 and At5g23020, have been identified at the GSL-ELONG locus,

which controls variation in the side-chain length of Met-derived aliphatic glucosinolates (Magrath et al., 1994; Campos de Quiros et al., 2000; Kroymann et al., 2001). These genes encode methylthioalkylmalate synthases 1 and 3 (MAM1 and MAM3), which catalyze the condensation of different methylthio-2-oxoalkanoic acids with acetyl-CoA to form 2-methylthioalkylmalates (Textor et al., 2004; Textor et al., 2007). This reaction is the initial step in the Met chain elongation cycle, the first part of the biosynthesis of aliphatic glucosinolates (see below). Thus MAMs take part in specialized metabolism.

In *Arabidopsis thaliana*, two other genes of the IPMS gene family encode true IPMS (de Kraker et al., 2007). Enzyme assays performed with purified recombinant IPMS1 (At1g18500) and IPMS2 (At1g74040) unambiguously demonstrate the function of these proteins in Leu biosynthesis and distinguished them from the MAM1 and MAM3. IPMS1 and 2 have nearly identical substrate specificities. They efficiently catalyze the condensation of 3MOB and acetyl-CoA into 2-isopropylmalate, while the formation of 2-methylthioalkylmalates occurs only at very low rates in the presence of these enzymes. In addition, almost no activity is observed with 3MOP and 4MOP (the 2-oxo acid intermediates of Ile and Leu).

Most biochemical properties of IPMS1 and IPMS2 are identical including the dependence on millimolar concentrations of Mg^{2+} for optimal enzyme activity and the affinities towards 3MOB (304 and 279 µM, respectively). However, a substantial difference is observed between the K_m values for acetyl-CoA, which is 45 µM for IPMS1 and 16 µM for IPMS2. In addition, both enzymes seem to differ in their higher order structure. IPMS1 tends to form a dimer while IPMS2 has been detected as a tetramer.

Minor differences were also seen between the amino acid profiles of *ipms1* and *ipms2* knockout lines. While no changes in the contents of Leu and most other amino acids were seen in any of the mutants in comparison to wild type, the knockout of IPMS1 leads to a slight increase of Val (Field et al., 2004; de Kraker et al., 2007). The absence of any detectable change in Leu content demonstrates that IPMS1 and 2 compensate for each other's absence, since it is unlikely that the very low IPMS activity of MAM1 or MAM3 substantially contributes to Leu biosynthesis. No significant changes were seen in the glucosinolate profiles of the *ipms* mutants, which is consistent with the very low rates seen in the activity tests with these enzymes and the substrates of Met chain elongation.

IPMS acts at the branch point between Val and Leu biosynthesis and is one of three feedback-regulated enzymes in the biosynthesis of BCAA (further details see below).

Isopropylmalate Isomerase

The second step in the Leu biosynthesis is catalyzed by isopropylmalate isomerase (IPMI) also known as isopropylmalate dehydratase (EC 4.2.1.33, Figure 2). In this reversible reaction, 2-isopropylmalate (2-IPM) is converted to 3-isopropylmalate (3-IPM). IPMI belongs to the family of aconitase, usually monomeric proteins containing [4Fe-4S] clusters (Gruer et al., 1997).

Two types of IPMIs have been identified in different organisms. In fungi, IPMI is a monomeric protein entirely present in the cytosol (Kohlhaw, 2003). In prokaryotic organisms, IPMI is a heterodimer composed of a large (*leuC*) and a small subunit (*leuD*), which align with the N-terminal part and C-terminal part of the monomeric IPMI from fungi (Gruer et al., 1997; Kohlhaw, 2003). Likewise, plants encode a heterodimeric IPMI. In *Arabidopsis thaliana* three genes for small subunits (At2g43090 (*IPMI SSU1*), At2g43100 (*IPMI SSU2*), and At3g58990 (*IPMI SSU3*) and one gene encoding a large subunit (At4g13430, *IPMI LSU1*) have been identified.

Very recently, the function of the different IPMI genes have been studied in various *Arabidopsis thaliana* mutants (Knill et al., 2009; Sawada et al., 2009). Different *ipmi lsu1* knockdown mutants show striking changes in the composition of Met-derived aliphatic glucosinolates (further details see below). In addition, Met, S-methylmethionine (SMM), a storage or transport form of Met, and other Met derivatives are increased. This metabolic phenotype unambiguously demonstrates the participation of this gene in the Met chain elongation required for the biosynthesis of aliphatic glucosinolates with variable chain lengths. However, there is also clear evidence for a function of this gene in Leu biosynthesis. Apart from an increase of Val in the strongest mutant *ipmi-lsu1-1*, the accumulation of 2-IPM in two different mutant lines is unambiguous proof that the large IPMI subunit also has a role in amino acid metabolism. Thus IPMI LSU1 has a dual function in both primary and specialized metabolism.

The strong knockdown in *ipmi lsu1-1* promotes a severe delay in development, a phenotype not observed in the other moderate knockdown lines. Abnormal plant growth related to impaired expression of IPMI-LSU1-1 was also observed in *Arabidopsis thaliana* accession Bur-0 under certain growth conditions (Sureshkumar et al., 2009). Here the expansion of a triplet repeat in the third intron of the gene leads to reduced transcript levels when plants were grown under moderate heat or high-light stress. The phenotype could not be reversed by adding Leu, demonstrating that the growth abnormalities are not a consequence of lowered levels of this amino acid. Likewise no changes in Leu content were observed in any of the *ipmi lsu1* knockdown mutants in the Col-0 background, including *ipmi lsu1-3*, which exhibits retarded development (Knill et al., 2009).

Less is known about the functions of the different small subunits, but the analysis of knockout mutants suggests that the different gene products have specialized functions. For IPMI SSU1, no homozygous knockouts could be obtained. Accordingly, in siliques of the corresponding heterozygous plants, the maturation of part of the seeds is aborted. This embryo-lethal phenotype is observed in two independent lines (SALK 115589 and SALK 111666) demonstrating that IPMI SSU1 is essential for seed development. This phenotype cannot be compensated by the other genes encoding small subunits suggesting a predominant role of this protein in Leu biosynthesis. However, further experiments are required to support this function (Knill et al., 2009).

Knockout mutants have also been analyzed for the other two genes encoding IPMI SSU2 and IPMI SSU3. Homozygous plants have been obtained for each of the genes. However, both amino acid and glucosinolate profiles exhibit only very moderate or no changes at all. These phenotypes suggest that IPMI SSU2 and IPMI SSU3 have redundant functions in the biosynthesis of Met-derived aliphatic glucosinolates (Knill et al., 2009).

Although further analysis of the small IPMI subunits is required to unambiguously determine their function, IPMI from

Arabidopsis thaliana and probably also from other Brassicaceae exhibits extraordinary features: the enzyme has a variable composition and the functions of the distinct heterodimers are most likely determined by the small subunits. The heterodimeric nature of the enzymes joins two evolutionary lineages. The large subunit has a dual function. It seems likely that this protein was originally exclusively active in amino acid metabolism and that it gained a new function in the specialized metabolism without the loss of its original function. Apparently the gene did not undergo duplication followed by specialization, a scenario, which seems to have occurred for the small subunits IPMI SSU2 and IPMI SSU3. These proteins seem to have lost their original function in Leu biosynthesis. This loss was only possible since IPMI SSU1 retained its original, essential function in 2-IPM isomerization required for the formation of Leu.

Isopropylmalate Dehydrogenase

The penultimate reaction in Leu biosynthesis, the oxidative decarboxylation of 3-isopropylmalate to 4-methyl-2-oxopentanoate (4MOP) is catalyzed by the isopropylmalate dehydrogenase (IPMDH, EC 1.1.1.85, Figure 2). This enzyme has been purified as a 52 kDa protein from pea and respective cDNAs have been cloned from rape seed and potato (Jackson et al., 1993; Singh, 1999). Later three *IPMDH*-like genes have been identified in the *Arabidopsis thaliana.* These have been designated AtIMD1 or AtIPMDH1 (At5g14200), AtIMD2 or AtIPMDH3 (At1g31180), and AtIMD3 or AtIPMDH2 (At1g80560). The cDNAs of these genes rescue the Leu auxotrophy of respective a yeast Leu2 mutant indicating their function as IPMDH (Nozawa et al., 2005).

Up to now little is known about IPMDH in *Arabidopsis thaliana*. Very recently, AtIMDH1 (AtIMD1, At5g14200) has been functionally characterized (He et al., 2009). This detailed study showed that the recombinant protein shows high activity with 3-IPM and that the level of free Leu is reduced in an *ipmdh1* knockout mutant. These findings demonstrate the function of this protein as isopropylmalate dehydrogenase, consistent with yeast complementation studies. But apart from its role in Leu biosynthesis the protein is also required for efficient biosynthesis of Met-derived aliphatic glucosinolates. Reduced levels of C4 and extremely low contents of C6-C8 glucosinolate species in both leaves and seeds of the *ipmdh1* knockout and of another knockdown mutant strongly suggest that IPMDH1 also catalyzes oxidative decarboxylation steps in the Met chain elongation cycle (He et al., 2009; Sawada et al., 2009). The role of this gene in secondary metabolism is corroborated by its perfect co-expression with crucial genes active in Met chain elongation including the common control by the major regulators of glucosinolate biosynthesis (Gigolashvili et al., 2007; Hirai et al., 2007; Sonderby et al., 2007). AtIPMDH2 and AtIPMDH3 did not display any changes in the glucosinolate profile suggesting that these genes might be primarily active in Leu biosynthesis (He et al., 2009).

Branched-chain Aminotransferase

The last step in the biosynthesis of BCAA is a transamination step converting the 2-oxo acids into the corresponding amino acids (Singh, 1999; Diebold et al., 2002; Schuster and Binder, 2005). These reactions are catalyzed by a branched-chain aminotransferase (BCAT, EC 2.6.1.42, also known as branched-chain amino acid transaminase), which can also initiate degradation of BCAA (see below). BCATs have been partially characterized in a number of species, including spinach, potato, barley, *Nicotiana sylvestris*, and melon (Gonda *et al*., ; Hagelstein *et al*., 1997; Campbell *et al*., 2001; Malatrasi *et al*., 2006; Gao *et al*., 2009). In *Arabidopsis thaliana*, seven *BCAT* genes have been identified, six of them being transcribed (Diebold et al., 2002). These six *BCAT* genes have been designated *BCAT1* (At1g10060), *BCAT2* (At1g10070), *BCAT3* (At3g49680), *BCAT4* (At3g19710), *BCAT5* (At5g65780) and *BCAT6* (At1g50110). So far no evidence has been obtained for the transcription of *BCAT7* (At1g50090). This gene is considered to be a pseudogene, although it encodes an intact open reading frame.

The cDNAs of five *Arabidopsis thaliana BCAT* genes restore growth of a yeast Δ*bat1*/Δ*bat2* double knockout mutant on a medium without BCAA confirming the biosynthetic BCAT activity of these plant genes. The sole exception is BCAT4, which does not restore auxotrophy for Ile and Val (Diebold *et al.*, 2002). GFP tagging experiments, mostly performed with cDNA fragments, covering the N-terminal parts of the various BCATs revealed that the gene products are transported to at least three distinct subcellular localizations (details see below). The compartmentalization of the distinct BCATs might explain the difficulties encountered in the characterization of BCAT activity in total cell extracts (Singh, 1999). An approach to enrich these proteins from spinach chloroplasts allowed the discrimination of two forms of BCAT activities: one was described as valine aminotransferase with a clear preference towards 3MOB, the other was described as leucine/isoleucine aminotransferase showing highest activities with 4MOP and 3MOP (Hagelstein et al., 1997).

According to GFP tagging experiments BCAT1 is a mitochondrial protein. As many enzymes of Leu degradation pathways are present this organelle, it is suggested that this enzyme initiates the degradation of this amino acid (further details see “Degradation of branched-chain amino acids in mitochondria”).

BCAT2 is located in plastids. Microarray studies showed that the *BCAT2* gene is basically expressed at a very low level, but transcript levels strikingly rise in response to various stresses and hormone treatments (Matsui *et al*., 2008). This also includes an increase of BCAT2 transcript levels after ABA treatment, which also elevates levels of free BCAAs (Urano *et al*., 2009). It was thus speculated that the contribution of BCAT2 to BCAA biosynthesis might be particularly strong under stress conditions. This potential function is supported by the observation that also transcription of methionine γ-lyase is up-regulated under stress conditions accompanied by increasing incorporation of carbon from Met into Ile (Jander and Joshi, 2010). However, promoter activity of BCAT2 is triggered also by carbon starvation, similar to promoters of genes involved in BCAA degradation (Schuster and Binder, 2005).

BCAT3 is also located in plastids. Recently the function of this gene and its protein product has been characterized in detail (Knill *et al*., 2008). This analysis revealed a dual function for this protein. As expected, the protein is involved in BCAA biosynthesis. *In vitro* assays with recombinant protein showed Michaelis-Menten kinetics with all branched-chain keto acids.

Highest affinities were measured with 4-methyl-2-oxopentanoate (4MOP, K_m: 0.14 ± 0.04 mM) and 3-methyl-2-oxopentanoate (3MOP, K_m: 0.14 ± 0.3 mM). V_{max} is twice as high for 4MOP (27.42 ± 2.09 µmol/min*mg) as for 3MOP (13.33 ± 0.68 µmol/min*mg). A substantially lower affinity and maximal velocity was measured for 3MOB, the 2-oxo acid of Val (K_m: 1.38 ± 0.10 mM, V_{max}: 14.79 ± 0.35 µmol/min*mg). These values are in the range of those found for 4-methylthio-2-oxobutanoate (MTOB, K_m: 1.92 ± 0.27 mM, V_{max}: 21.01 ± 0.93 µmol/min*mg). MTOB is the transamination product of Met. This 2-oxo acid is the first intermediate in the biosynthesis of Met-derived aliphatic glucosinolates. The activity of BCAT3 with this compound and also with 5-methylthio-2-oxopentanoate (MTOP) demonstrates the function of this enzyme in generating Met derivatives. The dual function of BCAT3 is confirmed by *in vivo* metabolite profiling of a corresponding *bcat3-1* mutant. Both the changes in the composition of aliphatic glucosinolates and the lowered level of Val confirm the involvement of BCAT3 in both primary and secondary metabolism (Knill et al., 2008).

BCAT4 is a promiscuous member of the BCAT protein family showing an atypical substrate specificity (Schuster *et al.*, 2006). Of the standard substrates only Leu and 4MOP are converted, while more or less no *in vitro* activity can be detected with Ile and Val and the corresponding 2-oxo acids. Instead, strongest activities were found with MTOB and MTOP, intermediates of the Met chain elongation pathway, the first phase of glucosinolate biosynthesis. Metabolite profiles of two T-DNA knockout mutants confirmed the atypical character of this enzyme. While BCAA levels did not show significant changes, the levels of Met and Thr were increased. In addition, SMM, which is hardly detectable in wild-type plants, accumulated to substantial amounts in the *bcat4* mutants. Consistent with these changes of free amino acids, the content of Met-derived aliphatic glucosinolates is reduced by 50% (Schuster *et al.*, 2006; Sawada *et al.*, 2009). In summary, these data unambiguously demonstrate that BCAT4 participates in biosynthesis of aliphatic glucosinolates. By contrast, it is unclear whether BCAT4 has any function in BCAA metabolism. The *in vitro* activity of this enzyme with Leu is accompanied *in vivo* by only a slight insignificant increase of this amino acid in the knockout mutants. Thus it remains unclear whether BCAT4 has any substantial influence on the level of Leu and its corresponding 2-oxo acid *in vivo*. It might be possible that the observed activity with Leu is simply a relic of the evolutionary origin of BCAT4.

BCAT5 and BCAT6 have yet not been characterized in detail. GFP tagging experiments, as well as a proteomic approach of plastid protein, suggest a localization of BCAT5 in chloroplasts, but another proteomic study identified this protein in mitochondria (Diebold *et al.*, 2002; Taylor *et al.*, 2004; Zybailov *et al.*, 2008). The BCAT5 gene is constitutively expressed at a moderate level. According to its localization in plastids it might be involved in BCAA biosynthesis but might also have a role in glucosinolate biosynthesis.

The subcellular localization of BCAT6 is somewhat unclear but the protein seems to reside in the cytosol (Diebold *et al.*, 2002). The function of the BCAT6 gene is completely unknown. It is basically expressed at very low levels but exhibits increased promoter activity in the shoot apex and in root tips (Schuster and Binder, 2005).

REGULATION OF BCAA BIOSYNTHESIS

In *Arabidopsis thaliana* three enzymes required for BCAA biosynthesis are under allosteric control: TD, AHAS and IPMS (Figure 2). TD forms a homo-tetramer of identical subunits with an approximate size of 60 kDa. Inhibition by Ile results from an increase in the K_m for the substrate Thr in the presence of Ile. The negative effect of this amino acid can be antagonized by Val. These effects seem to be accompanied by changes of the quaternary structure, whereby Ile induces dimerization and high Val concentrations restore the formation of tetramers (Halgand et al., 2002). Apart from that so-called ACT domains play a role in allosteric feedback regulation of BCAA biosynthesis. These small regulatory units have been shown to be involved in allosteric regulation of many proteins involved in amino acid metabolism (Grant, 2006). In TD, a regulatory unit is formed by the ACT motif, which serves as binding site for the amino acids. In *Arabidopsis thaliana* TD, the different ACT domains seem to form two non-equivalent binding sites, which implies that there are separate binding sites for Ile and Val. However, the interaction of these amino acids with the protein is more complicated. The binding of Ile to a site with high binding affinity provokes a conformational modification of the protein, which leads to binding of Ile to a second site. The interaction of Ile with this second site is needed for inhibition. Site-directed mutagenesis of *Arabidopsis thaliana* TD locus *OMR1*, producing Ile-insensitive TD mutants, revealed two regulatory regions R4 and R6 at the carboxy-terminal end that are needed to express full inhibition by Ile (Garcia and Mourad, 2004). Val competes with Ile for binding to the high affinity site, thereby preventing binding of Ile, which impedes inhibition.

Native (and *in vitro* reconstituted) AHAS is inhibited by each of the BCAAs. Synergistic effects were observed between Leu and Ile, and Leu and Val, with the latter showing particularly strong inhibitory effects. Inhibition is suggested to occur mainly by a decrease of the catalytic constant. Like TD, the regulatory subunit of AHAS contains ACT domains. AHAS retains a residual activity at saturation of inhibitor of about 30% (Curien *et al.*, 2008).

IPMS, the third enzyme of BCAA biosynthesis, is also under allosteric control. In this protein, the regulatory unit is also separated from the catalytic domain as observed for ACT containing enzymes, but the structure of the IPMS regulatory domain is not related to ACT. Instead, amino acid identities in the hydrophobic Leu binding pocket build an alternative structure which is conserved between IPMS from *Arabidopsis thaliana* and *Mycobacterium tuberculosis* (de Kraker *et al.*, 2007). IPMS acts at the branch point towards Leu and competes with BCATs for the common intermediate 3MOB. The enzyme is feedback regulated by Leu, thereby controlling the carbon flux into the biosynthesis of this amino acid (Hagelstein and Schultz, 1993; Singh, 1999; de Kraker *et al.*, 2007). The inhibitory potency of Leu was found to be substantially different for IPMS from spinach and *Arabidopsis thaliana*, which might be the result of different assay conditions (Curien *et al.*, 2008).

Whether additional regulatory mechanisms exist to control BCAA biosynthesis is presently unclear. Transcription of genes coding for enzymes of BCAA biosynthesis has been analyzed in different plant species. However, clear common patterns in terms of spatiotemporal transcription or responses to internal and external stimuli are not obvious.

FUNCTIONAL AND EVOLUTIONARY RELATIONSHIP OF LEUCINE BIOSYNTHESIS AND METHIONINE CHAIN ELONGATION

The above described reaction steps in the biosynthesis of Leu match reaction sequences found in several other pathways. This so called alpha-keto acid elongation (alphaKAE) pathway elongates aliphatic acids by single methylene groups as for instance 3MOB, which is elongated to 4MOP during Leu biosynthesis (Kroumova and Wagner, 2003). AlphaKAE can occur in basic pathways such as the tricarboxylic acid cycle and in more specialized reaction cascades like the synthesis of sugar-ester acyl acids in certain species of the Solanaceae and the Met chain elongation pathway, the first part in the biosynthesis of Met-derived aliphatic glucosinolates (Kroumova and Wagner, 2003; Halkier and Gershenzon, 2006). Glucosinolates, also called mustard oil glucosides, are specialized metabolites, which are found in species of the order of the Capparales but also in the completely unrelated genus *Drypetes*, which belongs to the family of the Euphorbiaceae (Mikkelsen et al., 2002; Wittstock and Halkier, 2002; Grubb and Abel, 2006; Halkier and Gershenzon, 2006).

Glucosinolates are categorized into Trp-derived indole, Tyr- or Phe-derived aromatic and aliphatic glucosinolates. Biosynthesis of aliphatic glucosinolates starts out from Ala, Val and Leu and but the most abundant group of aliphatic glucosinolates is synthesized from Met. Met-derived glucosinolates are also predominant in the model plant *Arabidopsis thaliana* (Windsor et al., 2005). The biosynthesis of Met-derived glucosinolates occurs in three phases (Figure 3). First, in the chain elongation phase the side-chain of Met is elongated by one or several methylene moieties. The three-step cycles finally yield Met derivatives like homo-Met, dihomo-Met and so on. Second, based on these Met derivatives, the core structure of the glucosinolates is established. Third, these parent glucosinolates undergo secondary modifications. In summary, these reaction sequences yield an enormous spectrum of different glucosinolate species (Wittstock and Halkier, 2002; Halkier and Gershenzon, 2006).

The biochemical parallels between Leu biosynthesis and the Met chain elongation pathway were realized several decades ago, when the general scheme of chain elongation was proposed based on *in vivo* feeding experiments using ^{14}C-labelled amino acids and [2-^{14}C]acetate (Chisholm and Wetter, 1964; Underhill et al., 1973). The Met chain elongation pathway is initiated by the deamination of Met to form 4MTOB (4-methylthio-2-oxobutanoate). This 2-oxo acid is extended by a three-step cycle each including condensation with acetyl-CoA, followed by the rearrangement of a hydroxy group in an isomerization reaction and oxidative decarboxylation. This reaction series is identical to the reaction sequence in Leu biosynthesis. These transformations result in chain elongated 2-oxo acid (i.e. 5-methylthio-2-oxopentanoate after a single cycle). This 2-oxo acid can now re-enter the pathway and undergo another round of elongation consisting of condensation, isomerization and oxidative decarboxylation, or it is transaminated back to a Met derivative (Figure 3).

The close relationship between the Met chain elongation and the Leu biosynthesis pathways is also evident on the genetic level. Initial evidence for this was obtained by mapping the quantitative trait locus GS-Elong controlling the chain length of Met-derived glucosinolates in *Arabidopsis thaliana* and *Brassica napus*. This genetic approach identified the two methylthioalkylmalate synthase genes *MAM1* and *MAM3*, formerly *MAML*. The *MAM1* gene product is responsible for a glucosinolate polymorphism between *Arabidopsis thaliana* ecotypes Col-0 and Landsberg *erecta* (L*er*) (Magrath et al., 1994; Campos de Quiros et al., 2000; Kroymann et al., 2001). This enzyme specifically catalyzes the condensation reaction of the first two Met elongation cycles. Accordingly, the occurrence of MAM1 in different *Arabidopsis thaliana* accessions (including Col-0) correlates with the preferential accumulation of glucosinolates that have passed two cycles of chain elongation (Kroymann et al., 2003; Textor et al., 2004). Other accessions, as for instance in L*er*, do not encode a functional *MAM1*, but instead posses the *MAM2* gene, which correlates with the predominant occurrence of glucosinolates that have undergone only a single round of elongation (Kroymann *et al.*, 2003). In contrast, the *MAM3* gene is present in all accessions investigated. The knock-

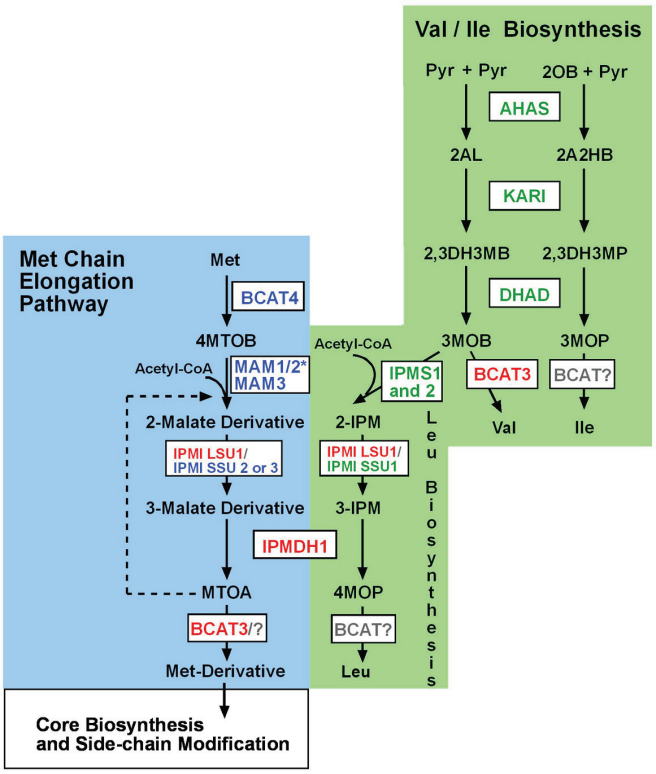


Figure 3. Branched-chain amino acid biosynthesis (shaded in green) and Met chain elongation pathway (shaded in blue). Enzymes are given in boxes and are abbreviated as given in the text. Proteins given in green are specific for BCAA biosynthesis. Proteins active in Met chain elongation are given in blue. Proteins active in both pathways are highlighted in red. MAM1/2* indicates that either MAM1 (in Col-0) or MAM2 (in Landsberg *erecta*) catalyze condensation reactions leading to the preferential accumulation of C4 (in Col-0) and C3 (in L*er*) glucosinolates.

Abbreviations of metabolites are: Pyr: pyruvate; 2OB: 2-oxobutanoate; 2AL: 2-acetolactate; 2A2HB: 2-aceto-2-hydroxybutanoate; 2,3DH3MB: 2,3-dihydroxy-3-methylbutanoate; 2,3DH3MP: 2,3-dihydroxy-3-methylpentanoate; 3MOB: 3-methyl-2-oxobutanoate; 3MOP: 3-methyl-2-oxopentanoate; 4MOP: 4-methyl-2-oxopentanoate; 2-IPM: 3-isopropylmalate; 3-IPM: 2-isopropylmalate; 4MTOB: 4-methylthio-2-oxobutanoate; MTOA: 4-methylthio-2-oxo acid.

out of this gene causes a complete absence of long chain aliphatic glucosinolates (Field et al., 2004). MAM3 is involved in the biosynthesis of Met-derived glucosinolates with side chains of all lengths (Textor *et al*., 2007).

In *Arabidopsis thaliana* the MAM synthases are highly similar to IPMS1 and 2, both types of enzymes catalyzing the same type of reactions, i.e. condensation of a 2-oxo acid with acteyl-CoA. These characteristics group MAM synthases and IPMS1 and 2 into a small protein family.

Likewise, all other proteins with predicted functions in Leu biosynthesis like IPMIs, IPMDHs and BCATs form small families in *Arabidopsis thaliana*. Like MAM and IPMS, members of these protein families are also involved in both Leu and/or glucosinolate biosynthesis.

As mentioned above, BCAT4 catalyzes the dedicated transamination reaction in the biosynthesis of Met-derived aliphatic glucosinolates (Schuster *et al*., 2006). From the standard substrates, BCAT4 only converts Leu and its 2-oxo acid 4MOP. Whether this *in vitro* activity is of any biological relevance is unclear, so it seems that BCAT4 gained a new function in glucosinolate biosynthesis and has completely lost its original function in the biosynthesis of BCAA. By contrast, BCAT3 exhibits a dual function being active in both biosynthesis of BCAAs and in elongation of Met (Knill *et al*., 2008). Likewise, a dual function has been suggested for IPMDH1, one of three potential IPMDH proteins (He *et al*., 2009; Sawada *et al*., 2009). These proteins have acquired new functions without loss of their original functions.

Both evolutionary scenarios are combined in the heterodimeric IPMI. In *Arabidopsis thaliana* this enzyme is composed of a large subunit encoded by a single gene and small subunit, encoded by three genes. The large subunit exhibits a dual function both in Met chain elongation and in Leu biosynthesis while the different small subunits have distinct specialized functions either in Leu biosynthesis or Met chain elongation (Knill *et al*., 2009). Though there is no final proof yet, the substrate preference of the dimer seems to be determined by the small subunits.

In summary, these data unambiguously demonstrate the very close evolutionary and functional relationship of the enzymes required for Leu biosynthesis and Met chain elongation. These observations strongly suggest that both pathways derive from a common ancestral reaction sequence.

DEGRADATION OF BRANCHED-CHAIN AMINO ACIDS IN MITOCHONDRIA

Animals including humans cannot synthesize BCAAs and must obtain these amino acids with their diet. Unlike biosynthesis, animals are capable of breaking down Val, Leu and Ile. A number of severe diseases linked to genetic disorders in BCAA degradation demonstrate the importance of the degradation process. In general, the breakdown of BCAA resembles the turnover of fatty acids by β-oxidation.

In *Arabidopsis thaliana* and other plant species, various enzymes and enzyme activities for degradation of BCAA have been detected and characterized, many of them being orthologous to their counterparts in animals. Most of the proteins involved in the breakdown of Leu are also localized in mitochondria. In contrast, some enzymes suggested to be involved in turnover of Val are located in the peroxisomes. The compartmentalization of these degradation pathways physically separates the BCAA breakdown from biosynthesis in chloroplasts.

Branched-chain aminotransferase

The breakdown of BCAA is initiated by transamination reactions catalyzed by BCATs. These enzymes convert Val, Leu or Ile into the corresponding 2-oxo acids 3MOB, 4MOP and 3 MOP (Figure 4). The mitochondrial localization of BCAT1 (At1g10060) suggests that this protein might catalyze the initial transamination reaction. Indeed, activity of recombinant BCAT1 has been observed with all BCAAs and their corresponding 2-oxo acids demonstrating the potential of this enzyme to catalyze the initial transamination (Schuster and Binder, 2005). But expression of this gene is not triggered by darkness or carbohydrate starvation as it is observed for almost all other genes encoding enzymes of this degradation pathway (see below). Instead *BCAT1* seems to be transcribed in almost all parts of the plants except roots with only minor transcriptional responses to external stimuli (http://jsp.weigelworld.org/expviz/expviz.jsp, https://www.genevestigator.com/gv/index.jsp). Curiously *BCAT2*, a chloroplast member of this gene family, exhibits increased transcription in response to carbohydrate deficit (Schuster and Binder, 2005). Moreover this gene is clearly co-expressed with many other genes of the Leu catabolism (Mentzen *et al*., 2008). In another study, BCAT5 has been detected in mitochondrial preparations from *Arabidopsis thaliana* cell suspension cultures but has also been found in chloroplasts (Diebold et al., 2002; Zybailov et al., 2008). So far

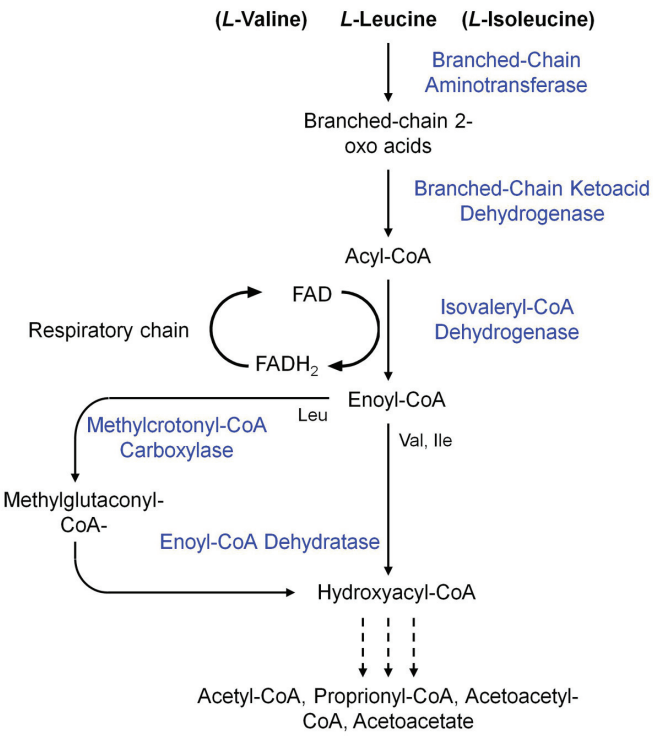


Figure 4. Key enzymes (blue) and metabolites (black) of branched-chain amino acid degradation.

the function of this enzyme has not been investigated in detail (Taylor et al., 2004). Thus it is presently unclear which BCAT is involved in BCAA degradation in *Arabidopsis thaliana*.

Branched-chain keto acid dehydrogenase

The second step in the breakdown of BCAAs, the irreversible oxidative decarboxylation of the branched-chain 2-oxo acids 3MOB, 4MOP or 3MOP into the acyl-CoA esters isobutyryl-CoA, isovaleryl-CoA or methylbutyryl-CoA, is catalyzed by the branched-chain keto acid dehydrogenase (BCKDH, EC 1.2.4.4, also called branched-chain keto acid dehydrogenase complex BCKDC, (Figure 4). BCKDH is a high molecular weight complex similar to pyruvate dehydrogenases both in mitochondria and chloroplasts and mitochondrial α-ketoglutarate dehydrogenase (Mooney et al., 2002). These sophisticated protein assemblies with masses up to 9 MDa consist of multiple copies of three subunits: E1, α-keto acid dehydrogenase (carboxylase); E2, a dihydrolipoyl acyltransferase and E3, a dihydrolipoyl dehydrogenase. E1, which is composed of E1α and E1β subunits, decarboxylates a 2-oxo acid using thiamine pyrophosphate as cofactor. E2 contains lipoic acid prosthetic groups. This subunit transfers the acyl groups to the reduced coenzyme A. E3 re-oxidizes the lipoyl groups of E2 with the concomitant formation of NADH. The core structures of the different 2-oxo acid complexes can have different forms. *In vitro* assembly of the *Arabidopsis thaliana* E2 core complex of BCKDH suggests an octagonal form. This complex consists of 24 subunits, which sum up to a molecular mass of 0.95 MDa (Mooney *et al.*, 2000)

In *Arabidopsis thaliana*, BCKDH subunits have been identified by tandem mass spectrometry and/or cloning of the corresponding cDNAs. E1α is encoded by At1g21400 (or At5g09300, identified by mass spectrometry, but the corresponding gene is not co-expressed with other genes of the Leu catabolism). E1β is encoded by At3g13450, E2 by At3g06850, and E3: by At3g17240 (Mooney et al., 1999; Fujiki et al., 2000; Taylor et al., 2004). In a heterotrophic cell suspension culture of this species BCKDH activity was detected with the 2-oxo acids of all three BCAAs with a slight preference for 3MOB and 4MOP. However, when O_2 consumption was measured in isolated mitochondria, considerable rates were only detected after addition of 3MOB, the 2-oxo acid of Val. When BCAAs were added to the isolated organelles, highest O_2 consumption was seen for Val, followed by Leu. Almost no consumption was observed with Ile (Taylor et al., 2004). These data suggest that Leu and Val are degraded in *Arabidopsis thaliana* mitochondria.

In mammals BCKDH, like the plant mitochondrial pyruvate decarboxylase, is regulated by phosphorylation of a conserved Ser residue in the E1 subunit. Whether a similar regulation mechanism exists for BCKDH from plants is unknown at present (Mooney et al., 2002).

Isovaleryl-CoA dehydrogenase

In the next step the different acyl-CoA esters are oxidized by an acyl-CoA dehydrogenase. Proteins with high similarities to isovaleryl-CoA dehydrogenases (IVD, EC 1.3.99.10) have been identified in *Arabidopsis thaliana* (At3g45300), potato and pea, but the substrate specificities vary between these proteins from different species (Däschner et al., 1999; Reinard et al., 2000; Däschner et al., 2001; Faivre-Nitschke et al., 2001) (Figure 4). The pea IVD is active exclusively with isovaleryl-CoA (Reinard et al., 2000), which is also the best substrate for the enzyme from *Arabidopsis thaliana* (Däschner et al., 2001). This is consistent with an *in vivo* role of IVD in the turnover of Leu. In addition, recombinant IVD from *Arabidopsis thaliana* has substantial *in vitro* activity with isobutyryl-CoA, an intermediate of Val degradation. This substrate preference fits well with the above described observation that O_2 consumption of isolated *Arabidopsis thaliana* mitochondria is stimulated by addition of Val and Leu. Very recently it was found that IVD is not only involved in BCAA degradation, but also plays an important role in the breakdown of phytol and Lys. These functions seem to be critical for alternative respiration under dark-induced senescence (Araujo et al., 2010).

In contrast to *Arabidopsis thaliana*, where only a single *IVD* gene is present, two genes (*StIVD1* and *StIVD2*) have been found in potato (Faivre-Nitschke et al., 2001; Goetzman et al., 2005). StIVD2 exhibits the same substrate specificity as the *Arabidopsis thaliana* protein, while StIVD1 shows strongest activity with 2-methylbutyryl-CoA. This is a degradation intermediate of Ile and StIVD1 was accordingly designated 2-methylbutyryl-CoA dehydrogenase. However, potato might be a special case among plants since no homologue of this gene has been found in other plant species (Goetzman et al., 2005).

Recently a screen for *Arabidopsis thaliana* mutants defective in accumulation of free amino acids in seeds identified a line that exhibits elevated levels of twelve standard amino acids (Gu *et al.*, 2010). Among those, BCAAs exhibit particularly high levels (Leu, Ile and Val are increased 28-, 30- and 16-fold). Therefore the mutant was named HBC for high branched-chain amino acids. But apart from the elevated proteinogenic amino acids, also homomethionine and the homomethionine-derived 3-isovaleroyloxypropylglucosinolate accumulated to increased levels. The different metabolic phenotypes were interpreted to be related to isovaleryl-CoA dehydrogenase activity and indeed inactivated IVD was found to cause the changes in the metabolite profile. These data nicely indicate another metabolic link between BCAA catabolism and the specialized metabolism, which corroborates the close link between BCAA, Met and glucosinolate metabolism.

Electron-transfer Flavoprotein: Ubiquinone Oxidoreductase

IVD catalyzes the transformation of isovaleryl-CoA into 3-methylcrotonyl-CoA. The electrons of this redox reaction are transferred into the respiratory chain at the level of ubiquinone via FAD, the electron-transfer flavoprotein (ETF) and an electron-transfer flavoprotein: ubiquinone oxidoreductase (ETFQO) (Figure 4). In *Arabidopsis thaliana* both subunits of the ETF have been identified in mitochondria by mass spectrometry (ETFα: At1g50940 and ETFβ: At5g43430) (Taylor et al., 2004). These proteins have not been specifically analyzed in plants, but ETFα have recently been found to bind Cu2+ (Tan et al.).

Much more is known about ETFQO (EC 1.5.5.1, At2g43400) (Ishizaki et al., 2005). *ETFQO* is highly expressed in senescent and sucrose-starved plants and cell cultures as well as in

plants grown in continuous darkness. In line with this expression pattern, mutants lacking this gene exhibit a strong phenotype when cultivated under such conditions. This includes impaired development of female gametophytes and signs of accelerated senescence. This is accompanied by alterations in metabolite profiles with two major changes. First, under these growth conditions, chlorophyll content declines more rapidly in the *etfqo* mutants. This is accompanied by a dramatic increase of phytanoyl-CoA, suggesting a connection of the ETFQO function with the degradation of chlorophyll. Second, the distinct increase of isovaleryl-CoA in the mutant indicates that this protein is part of the electron transfer cascade from IVD into the respiratory chain. No accumulation of isobutyryl-CoA and 2-methylbutyryl-CoA has been detected in *etfqo* mutants. This metabolite pattern suggests that this protein is involved in the degradation of Leu, however, the accumulation of several amino acids including Val and Ile might indicate that ETFQO is also needed for degradation of other amino acids.

Methylcrotonyl-CoA carboxylase

After oxidation of acyl-CoA esters by IVD the Leu degradation proceeds via a carboxylation step catalyzed by methylcrotonyl-CoA carboxylase (MCCase, EC 6.4.1.4, Figure 4) to yield 3-methyl-glutaconyl-CoA. MCCase is a biotin containing heterodimer and was actually the first enzyme described in the plant mitochondrial Leu degradation pathway (Alban et al., 1993; Song et al., 1994). It was detected as a protein accumulating under carbohydrate starvation, whose expression is induced in darkness (Aubert et al., 1996; Che et al., 2002). In *Arabidopsis thaliana*, At1g03090 and At4g34030 encode the α (MCCA) and β (MCCB) subunits of MCCase. Both genes are clearly co-expressed with other genes for the Leu catabolic pathway (Mentzen et al., 2008).

Enoyl-CoA Hydratase

The next step in Leu degradation is catalyzed by an enoyl-CoA hydratase-like protein (Figure 4). In *Arabidopsis thaliana* two proteins with similarity to enoyl-CoA hydratases (EC 4.2.1.17) are predicted to be imported into mitochondria (At3g60510 and At4g31810), the latter having also been detected in a proteomic approach (Millar et al., 2001). Both proteins could potentially be involved in the mitochondrial breakdown of Leu, as well as Ile and Val. However, expression patterns of these genes do not correlate with those of other genes coding for known components of the Leu catabolic pathway.

The reactions catalyzed by enoyl-CoA hydratase result in the formation of different hydroxy-acyl-CoAs. After this step the further catabolic cascade splits into separate pathways with different enzymes, some of which are detected in mitochondrial or peroxisomal fractions, while others are predicted to be imported into one of these organelles or have unclear subcellular localizations (Reumann et al., 2004; Taylor et al., 2004; Eubel et al., 2008; Reumann et al., 2009). These proteins have not yet been analyzed in *Arabidopsis thaliana* or any other plant species.

BCAA ARE DEGRADED IN PLANT MITOCHONDRIA AND/OR PEROXISOMES

An important pending question is whether all three BCAA are indeed degraded in *Arabidopsis thaliana* or in other plants. As outlined above there is compelling evidence for the turnover of Leu. All enzymes required for the degradation of this amino acid down to 3-methyl-glutacomyl CoA are located in mitochondria (Anderson et al., 1998). Candidate enzymes for further steps have been identified but not yet analyzed. More studies are required for the unequivocal proof of the degradation of Ile and Val. For the latter degradation in peroxisomes has been suggested several decades ago (Gerbling and Gerhardt, 1989) and more recently substantiated by the analysis of a peroxisomal short-chain acyl-CoA dehydrogenase (Hayashi et al., 1999) and β-hydroxyisobutyryl-CoA hydrolase (Lange et al., 2004). However, it seems that a full set of enzymes required for a complete breakdown of this amino acid is not present in this organelle. Thus the pathways for degradation of BCAAs might be distributed over several subcellular compartments with mitochondria and peroxisomes being most important for the turnover of Leu and Val.

REGULATION OF MITOCHONDRIAL BCAA DEGRADATION

In *Arabidopsis thaliana* BCKDH subunits E1β and E2, IVD, both MCCase subunits and of ETFQO are highly expressed in darkness or under carbohydrate starvation. This observation clearly indicates an important function of the BCAA catabolism under such environmental conditions. This conclusion is supported by the analysis of *etfqo* and more recently of *ivd* knockout mutants, which show an accelerated senescence phenotype under cultivation in extended darkness (Ishizaki et al., 2005; Araujo et al., 2010). In addition, co-expression mining tools consistently show that these genes form a co-expression network (Mentzen et al., 2008). Unfortunately, co-expression studies revealed only a single candidate factor (At5g49450, bZIP family transcription factor) that might be involved in regulation of these genes, though expression correlation might only be a vague hint for this function. Thus, more or less nothing is known about regulation of BCAA degradation.

FUTURE PROSPECTS

Almost all enzymes in the biosynthesis and many enzymes in the degradation of BCAAs have been characterized in *Arabidopsis thaliana*. However, so far little is known about transcriptional regulation of the genes coding for these enzymes. Since *Arabidopsis thaliana* and other plant species are capable of both *de novo* BCAA biosynthesis and degradation, these counteracting pathways have to be carefully balanced to maintain the functionally required homeostasis of this important group of amino acids. Unraveling the underlying regulatory mechanisms of BCAA metabolic pathways will be one of future challenges and *Arabidopsis thaliana* with its extraordinary advanced scientific facilities will be the ideal platform for these future studies.

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